

PATTERNS AND CONTROL OF NEUROSECRETION FOR THE CYTOCHROMOGENIC HORMONE IN *BLABERUS DISCOIDALIS* COCKROACHES

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SUMMARY

1. Corpora cardiaca (CC) from immature and adult *Blaberus discoidalis* Serville of both sexes contained approximately equal amounts of activity for the cytochromogenic hormone (CGH), a neurohormone which stimulates cytohaem synthesis in fat body mitochondria on day 4 of adult life.

2. CGH activity remained at a constant level in the CC of males during days 0 and 1 of adult life, decreased during days 2 and 3, and then returned to the original level by day 4. Female CC followed a similar pattern except that the period of low CGH activity occurred 1 day later.

3. Variations in early adult feeding patterns or in daily photoperiod did not change the secretory pattern of CGH. However, injections of juvenile hormone III stimulated a precocious release of CGH on days 0–1. Thus, CGH and juvenile hormone may function in this insect as a coordinated endocrine sequence directing metabolic maturation of the fat body during early adult life.

INTRODUCTION

Hormones influence aspects of mitochondrial development in animal tissues. For example, treatment of euthyroid rats with thyroid hormone increases the synthesis of liver cytochrome *c* by 5.5 times (Booth & Holloszy, 1975). The synthesis of protein subunits for the apoenzyme of mitochondrial cytochrome oxidase, especially the 45 000 and 28 500 Da subunits, is selectively enhanced in hepatocytes from triiodothyronine-treated rats (Nelson *et al.* 1980). Growth hormone administration to hypophysectomized rats restores to control levels both the low cytochrome content and the low protein turnover rate in mitochondria of hepatocytes (Maddaiah, Weston, Chen & Collipp, 1976; Maddaiah, Collipp, Lin & Duffy, 1976). In insects, Keeley (1972) finds a neuroendocrine-dependent increase in mitochondrial respiratory capacity in the fat body of the cockroach, *Blaberus discoidalis* Serville. Development of the respiratory capacity in adult fat body mitochondria is inhibited by removal of the corpora cardiaca (CC), an insect neurosecretory structure. Conversely, injections of CC extracts stimulate full respiratory development in the fat body mitochondria. A general increase in the cytochrome content of the fat body mitochondria occurs during

the first 6 days of adult life (Keeley, 1977) and correlates with the increase in mitochondrial respiratory capacity (Keeley, 1972). Presumably, the neuroendocrine effect is related to the cytochrome synthesis.

Our laboratory has been using the *B. discoidalis* system as a simple model to investigate the regulatory role of hormones on aspects of mitochondriogenesis, especially cytochrome synthesis. An essential aspect of cytochrome biosynthesis is the production of the appropriate haem group for incorporation into the cytochrome apoprotein to produce the functional holocytochrome. To determine the role of neurohormones on cytochrome synthesis during mitochondriogenesis in the insect fat body, studies were undertaken on haem synthesis. A neuroendocrine-dependent, three- to five-fold increase in the synthetic rate for fat body cytohaems *a + b* occurs at 4–6 days of adult age in *B. discoidalis* (Keeley, 1978). Recently, we demonstrated that a small, hydrophobic peptide occurs in the CC of *B. discoidalis* and stimulates the increase in fat body cytohaem *a + b* synthesis (Hayes & Keeley, 1981). This peptide is associated with a neurosecretory granule fraction isolated from the CC (Smith, Hayes & Keeley, 1983), and we have designated this factor as the cytochromogenic hormone (CGH). In the present study, we determined the levels of CGH activity in the CC with respect to sex and age of adult *B. discoidalis*.

In addition to CGH content in the CC, the present studies examined the factors that may influence the timing of CGH secretion. A number of environmental and intrinsic factors influence the secretion of insect neurohormones. Long photoperiods (15–18 h) activate prothoracicotropic hormone secretion for diapause termination in pupae of *Antheraea pernyi* (Williams & Adkisson, 1964). A blood meal stimulates release of the egg development hormone to initiate ovarian development in anautogenous *Aedes taeniorhynchus* mosquitoes (Lea, 1970). In *Calliphora erythrocephala*, the tanning hormone, bursicon, is not released until the newly-emerged adults have burrowed free from the substrate in which they were buried during pupation (Fraenkel, 1936). Finally, both juvenile hormone (JH) and 20-hydroxyecdysone influence neurosecretory activity (Agui & Hiruma, 1977*a,b*; Hiruma, Yagi & Agui, 1978; Marks, Ittycheriah & Leloup, 1972; McCaffery & Highnam, 1975; Thomsen & Lea, 1969).

In the studies reported here, we determined that CGH was present in the CC of both sexes of adults and in nymphs. Adult males had a distinct secretory pattern for CGH early in adult life that appeared related to JH exposure.

METHODS AND MATERIALS

Experimental animals

Experimental animals were *Blaberus discoidalis* cockroaches reared in wood shavings at 27°C in a 12 h light: 12 h dark circadian cycle with dog food and water provided *ad lib*. Animals were segregated by sex and aged from the day of adult emergence (= day 0). Animals used to determine the time of day for CGH release were selected within 8 h of emergence on day 0.

Preparation of gland and tissue extracts

We decapitated appropriately-aged animals without anaesthesia and removed the CC from the head as described by Hayes & Keeley (1981). It should be pointed out

that hormone extracts are prepared only from CC of unanaesthetized animals since CC from CO₂-anaesthetized animals frequently have little or no CGH activity. We believe that the stress of CO₂ anaesthesia causes erratic neurosecretion. Isolated CC were washed and homogenized in Ephrussi-Beadle Ringer (EBR) (Ephrussi & Beadle, 1936), and the homogenate was frozen and thawed three times to disrupt neurosecretory granules. After centrifugation at 10 000 *g* for 5 min, the supernatant was removed and stored at -20°C. We prepared coxal muscle extracts in a similar manner for use as control injections. The protein concentration of the muscle extract was adjusted by dilution with EBR so that it was similar to that of a typical CC extract.

CGH bioassay

CGH activity was assayed in decapitated animals by intrahaemocoelic injections of the appropriate CC extract on days 2, 3 and 4 of adult life (Hayes & Keeley, 1981). Bioassay animals are anaesthetized with CO₂ to stop the heart beat prior to decapitation and thus minimize bleeding which interferes with proper sealing of the neck wound with a beeswax/petrolatum (50:50) mixture. Decapitation must be done immediately after the animal is anaesthetized and before the heart resumes pumping. The timing of the decapitation is critical to prevent endogenous CGH from escaping from the head and into the rest of the bioassay animal. CGH stimulation of cytohaem synthesis was measured on day 4 based on the rate of *in vivo* incorporation of ¹⁴C-aminolaevulinic acid (¹⁴C-ALA) into mitochondrial cytohaems *a* and *b*. Fat body mitochondria were isolated by our high-speed procedure (Keeley, 1973), and cytohaems *a* and *b* were extracted from isolated mitochondria with acidic acetone according to the method of Basford, Tisdale, Glenn & Green (1957).

Relative CGH activity titres

Relative activity titres for CGH were determined in CC from nymphs and adults by comparing dilutions of test CC with the dose-response curve derived from CC of 1-day-old adult males (Hayes & Keeley, 1981). From the dose-response curve, 0.05 CC (total dose) causes a half maximal response and >0.08 CC causes a full response.

Reagents

JH-III was obtained from Sigma Chemical Co., St Louis, MO. ¹⁴C-ALA was purchased from Research Products International, Mt Prospect, IL. All other chemicals were either commercial reagent grade or analytical grade.

RESULTS

CGH activity in the corpora cardiaca relative to sex and growth stage

We determined the relative amount of CGH activity at 1 day after ecdysis in the CC of nymphs and both sexes of adult *B. discoidalis*. The CGH titre in the test CC was compared at 0.05, 0.5 and 1.0 CC doses to our bioassay curve for the 1-day-old adult male, reference CC. All the test CC gave the same degree of response regardless of sex or growth stage (Table 1). Thus, within the limits of our bioassay, we determined that during the first day after ecdysis, the CGH activity was essentially identical in the CC from adults of both sexes and from last instar nymphs.

Table 1. *CGH activity in corpora cardiaca (CC) from nymphs and adult males and females of Blaberus discoidalis at 1 day after ecdysis*

Injection group tested	Total CC dose	CGH activity*
Muscle injection	None	212 ± 21 (8)
Nymph CC	1 CC	511 ± 95 (4)
	0.05 CC	303 ± 13 (7)
Male CC	1 CC	560 ± 72 (8)
	0.05 CC	372 ± 49 (8)
Female CC	0.5 CC	500 ± 53 (8)
	0.05 CC	382 ± 56 (8)

*CGH activity is expressed as d.p.m. mg⁻¹ mitochondrial protein h⁻¹.
Values are mean ± s.e. with the number of replicate animals tested shown in parenthesis.

Secretory patterns for CGH from adult corpora cardiaca

We measured the relative CGH-activity titre at various ages in the glands of male and female adult *B. discoidalis*. Day-0 CC from adult males had a level of CGH activity equal to day-1 CC (Fig. 1A). CGH activity decreased in the CC on days 2 to

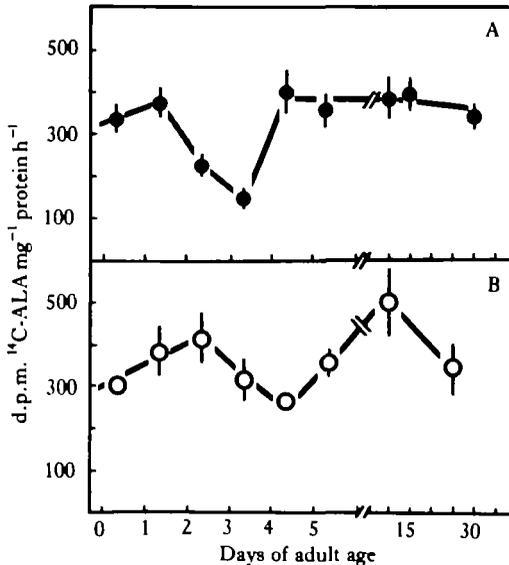


Fig. 1. Age-related patterns for CGH activity in corpora cardiaca of adult *Blaberus discoidalis*. All data points represent the time of CC removal relative to adult ecdysis (= day 0). CC extracts were prepared and injected into the standard bioassay system at a total dose of 0.05 CC. Each data point is the mean of eight replicate animals. Vertical lines indicate ± s.e. (A) *Male donor animals*: statistical analysis of bioassay results by Duncan's multiple range test shows that glands from days with the same letter do not result in significantly different cytochromogenic responses at $P < 0.05$ (day 0, ab; 1, a; 2, bc; 3, c; 4, a; 5, ab; 15, a; 30, ab). (B) *Female donor animals*: statistical analysis of bioassay results by Friedman's analysis of variance and Duncan's multiple range test show that glands from days with the same letter do not result in significantly different cytochromogenic responses (day 0, ab; 1, ab; 2, a; 3, ab; 4, b; 5, a; 10, a; 25, ab). The Duncan's multiple range indicates a significant difference to $P < 0.05$. ¹⁴C-ALA, ¹⁴C-aminolaevulinic acid.

3 and returned to the 1-day level by the afternoon of day 4. As much as 0.25 CC from 2- to 3-day-old animals still did not cause a response. The decline in CGH activity in the CC suggested that CGH was secreted in adult males between 2 to 4 days of age.

Adult females showed a similar age-related pattern for the levels of CGH activity in their CC (Fig. 1B). However, the time of CGH depletion in females occurred 24 h later, with minimum CGH activity in the CC around the afternoon of day 4. CGH activity levels fluctuated more during adult life in females than in males as indicated by the differences observed on days 15 and 30.

The reduction in the levels of CGH activity for male CC on days 2 and 3 could result from reasons other than neurosecretion (e.g. conversion of CGH to inactive forms in the CC, transport of CGH to another site, or inhibition of the bioassay by other chemical factors present in CC extracts on days 2 and 3). To eliminate these potential artifacts, decapitation experiments were performed to determine the timing of CGH secretion as based on the presence or absence of the normal 4-day increase in fat body cytohaem synthetic capacity. Once an animal is decapitated, the CGH source is eliminated, and the normal CGH-dependent increase in fat body cytohaem synthesis does not occur. Therefore, CGH must be released prior to the time of decapitation in order for the normal three- to five-fold increase in fat body cytohaem synthesis to occur on day 4.

We decapitated young adult males at various ages and measured their capacity for cytohaem $a + b$ synthesis at 4 days of adult age to determine the timing for the neurosecretion of CGH. If animals were decapitated prior to 33 h of adult age, no increase in cytohaem synthesis was observed (Fig. 2A). Cytohaem synthetic capacity reached only intermediate levels when animals were decapitated between 49 and 62 h of adult age. Decapitation of animals after 73 h of adult age resulted in a fully stimulated cytohaem synthetic capacity on day 4. These results confirmed that CGH secretion from the CC occurred in males during the middle of day 2 of adult age.

Environmental effects on CGH secretion

Since CGH secretion was timed to start precisely during the middle of day 2 in males, it was logical to assume that the animal received some type of uniform stimulus that initiated neurosecretory activity. Photoperiod 'counting' or the initiation of feeding activity were the most obvious candidates as regulatory stimuli for CGH neurosecretion.

Photoperiod effects were determined by keeping male animals in either constant light or constant dark during the first 4 days of adult life. We decapitated the animals at various ages during their constant photoperiodic regimen to determine the timing of CGH release based on the premise of the previous experiment. The occurrence of the natural, 4-day peak of fat body cytohaem synthesis was used to determine if CGH was released on schedule.

No photoperiod-related effect was found on the timing of CGH release in adult males (Fig. 2B). All test animals decapitated on or after the afternoon of day 2 exhibited the normal, day-4 peak of fat body cytohaem synthesis regardless of their photoperiodic regimen. Manipulation of the photoperiod neither advanced nor delayed the timing of CGH secretion.

A similar decapitation experiment was done on adult male animals that could not

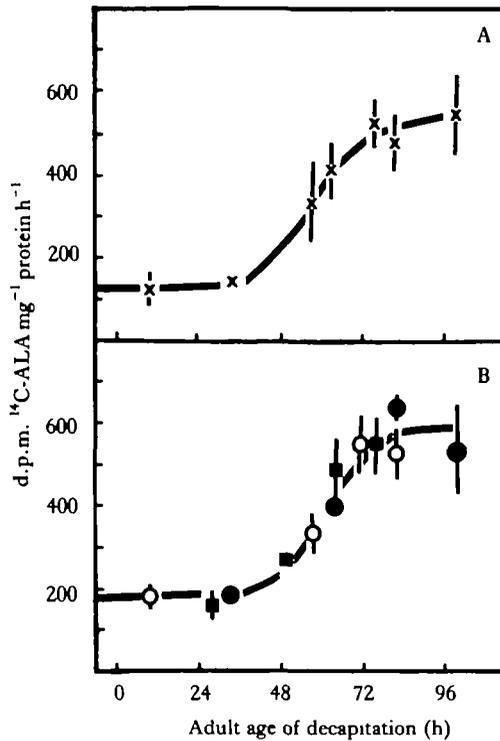


Fig. 2. The effects of age of decapitation on cytohaem synthesis in the fat body of 4-day-old adult male *Blaberus discoidalis*. Each point represents the mean for eight replicate test animals. Vertical lines indicate \pm s.e. (A) Males untreated until time of decapitation. (B) The influence of (○) constant light conditions, (●) constant dark conditions and (■) oral blockage on the release of CGH from the head.

consume food and water and were unable to move their mandibles. The oral blockage was obtained by gluing the mandibles shut with cyano-acrylate glue. As with photoperiod, inability to feed or chew had no effect upon the natural secretory pattern for CGH (Fig. 2B).

JH-III effects on CGH secretion

Since the environmental factors tested had no effect upon CGH secretion in adult male animals, we explored the possibility that CGH secretion responded to an internal signal. CGH release may occur as part of a preprogrammed sequence of endocrine events associated with moulting. Such a preprogrammed series of events is reported in *Manduca sexta* between ecdysteroids, eclosion hormone and bursicon (Truman, 1981).

We considered the possibility that JH may be related to CGH secretion in adult *B. discoidalis*. JH-III is present in adult female *B. discoidalis*, and its secretion begins around day 2 (I. M. Seligman & G. Bhaskaran, personal communication). JH-III serves as a gonadotropic hormone in female *B. discoidalis* and must be secreted independently of feeding since starved female *B. discoidalis* initiate oocyte maturation (McKercher, 1981). In male *B. discoidalis*, the corpora allata (CA) enlarge during the

first 3 days of adult life (L. L. Keeley, unpublished observations), and enlargement of the CA is an indicator for JH secretion (Szibbo & Tobe, 1981). Therefore, we speculated that increasing JH titres may stimulate CGH release during the early life of adult males. We tested the effect of administering exogenous JH-III on CGH secretion to explore the possibility of a JH-CGH endocrine interaction (Fig. 3).

If JH regulates CGH secretion, then injections of JH-III prior to the time for natural JH secretion should stimulate a precocious release of CGH from the CC. Early CGH release would be detected if test animals were decapitated late on day 1 prior to the time for natural CGH secretion and increased rates for cytohaem synthesis were still found in the day-4 assay. Several preliminary experiments were necessary to confirm the response of the bioassay animals to the various hormone regimens needed for this experiment.

First, JH-III was administered to test insects to see if it was stimulatory to fat body cytohaem *a + b* synthesis in the same manner as CGH. We injected a total dose of 3.0 μg of JH-III (1 μg daily in 5 μl of mineral oil) into decapitated bioassay animals using the normal, CGH-injection regimen. JH-III failed to stimulate day-4 fat body cytohaem synthesis (Fig. 3). This confirmed that JH-III *per se* had no direct cytochromogenic effects on the fat body by our usual injection regimen and bioassay.

Next, it was necessary to confirm that decapitated bioassay animals were capable of responding to precocious CGH exposure on days 0 and 1 of adult life with an increase in fat body cytohaem synthesis on day 4. Test animals that were decapitated

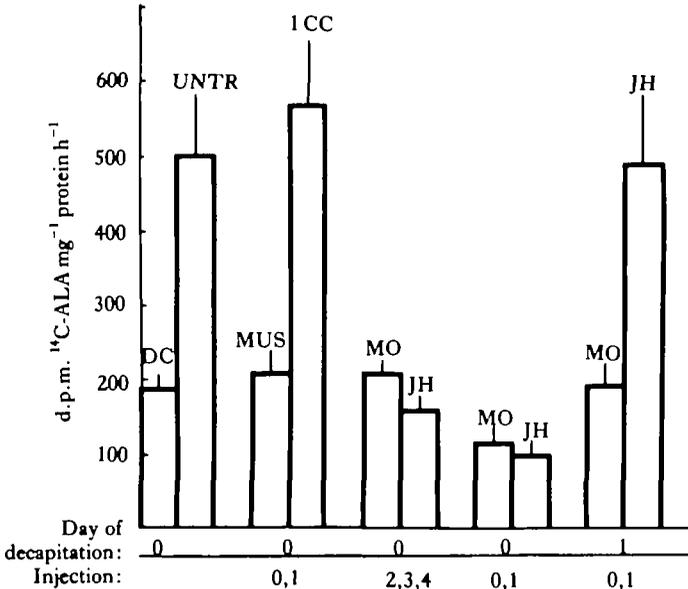


Fig. 3. The effects of juvenile hormone III on CGH secretion in adult male *Blaberus discoidalis*. Test animals were treated as indicated then decapitated at the age shown. Fat body cytohaem synthesis was assessed at 4 days of adult age. (DC), decapitation only; (UNTR), untreated animals provided with food and water *ad lib.*; (MUS), injected with coxal muscle extract; (1 CC), injected with male CC extract (1 CC total dose); (MO), injected with mineral oil; (JH) injected with JH-III (1 μg total dose). Histograms denote the mean of six to nine replicate test animals, and centred vertical lines indicate +s.e.

within 2 h of adult eclosion and injected with CC extracts on days 0 and 1 showed the normal increase in levels of cytohaem synthesis in the day 4 bioassay (Fig. 3). This confirmed that the premature presence of CGH on days 0 and 1 was stimulatory to cytohaem synthesis on day 4. Therefore, if JH-III initiated precocious CGH secretion from the CC on days 0 or 1, the effect of the secreted CGH would be detected by the bioassay. Conversely, animals decapitated within 2 h of adult eclosion and injected with 0.5 μg of JH-III on days 0 and 1 showed no elevation in cytohaem synthesis on day 4. Hence, JH-III did not have a direct effect upon fat body cytohaem synthesis when injected in this manner.

JH-III injections appeared to stimulate the early neurosecretion of CGH in adult males. JH-III (0.5 μg in 5 μl of mineral oil) was injected into normal animals at less than 4 h of adult age and again 24 h later. These early injections of JH-III were followed by decapitation at 34–36 h of adult age before the start of natural CGH release, which we estimated occurred at approximately 49 h of adult age. The result was that fat body cytohaem synthesis was at its normal, maximal level of activity on day 4, despite the fact that the head was removed before the time when CGH was secreted normally (Fig. 3). Animals injected with 5 μl of mineral oil at 4 and 24 h, then decapitated at 34–36 h, showed only the low cytohaem synthetic activity that is characteristic for decapitated animals. Therefore, the data demonstrate that, after JH-III injection, CGH was secreted 12–24 h prior to the time for its natural neurosecretion. This suggests that an increasing JH-III titre may be the stimulus that elicits the release of CGH during early adult life in *B. discoidalis* cockroaches.

DISCUSSION

The present results indicate that the timing of CGH secretion from the CC of *B. discoidalis* correlates with earlier studies on the time for the onset of active cytohaem synthesis in the fat body. The decapitation and relative titre studies demonstrated that CGH release from the CC started at about 49 h of adult life in the male and around 24 h later in the female. Maximum fat body cytohaem synthesis occurs at 4 days of adult life in males (Keeley, 1978), and it takes 36–48 h of CGH exposure for the fat body to attain its optimal capacity for cytohaem synthesis (Hayes & Keeley, 1981). Thus, the present findings confirm our two, earlier independent reports and indicate that CGH secretion starts during day 2 and results in the peak capacity for fat body cytohaem synthesis that is reached 48 h later, on day 4. Since the cytochrome content is increasing in the fat body mitochondria between days 2 and 6 (Keeley, 1977), it appears that CGH stimulates cytohaem synthesis for the purpose of increasing mitochondrial cytochrome formation.

The exactness of the time for CGH release suggests that CGH neurosecretion is a regulated process. However, our studies indicate that neither photoperiod nor feeding affect the time for CGH secretion in males. Photoperiod cycles and feeding are two extrinsic stimuli that frequently influence neuroendocrine activity (Williams & Adkisson, 1964; Mordue, 1967; Highnam & Mordue, 1974; Friedel & Loughton, 1980). Instead of extrinsic factors, our data suggest that a rising JH titre may stimulate the release of CGH in males.

The relationship between JH and the secretion of CGH appears to be similar to the

preprogrammed endocrine sequence (ecdysone, eclosion hormone, bursicon) associated with moulting in *M. sexta* (Truman, 1981). Each of these latter three hormones is responsible for specific physiological and behavioural events which are precisely timed to coordinate the successful completion of the moult. Moulting events occur in an obligatory sequence, and each event is regulated by a hormone that may also affect the release of the next hormone in the sequence. Similarly, after the adult moult in *B. discoidalis*, JH and CGH may both regulate aspects of adult fat body maturation in a sequence of coordinated endocrine events.

The precise timing for CGH secretion argues that the action of CGH is essential to the functioning and well-being of the organism. However, the particular physiological significance of a neuroendocrine regulation of mitochondriogenesis remains obscure. Although CGH is present in the CC of nymphs, we do not know at this time whether the CGH is secreted or whether it plays a role in the physiology of the immature instars. In adult male *B. discoidalis*, CGH appears related to an increase in biosynthetic activity of the fat body. There is a fluctuation in the content of stored metabolites in the fat body during the first 4 days of adult life (Mannix & Keeley, 1980), and new mitochondrial structures appear during this time (Keeley, 1981). It is midway through this 4-day maturation period that CGH is secreted so that cytohaem production reaches a maximum at the end of cytoplasmic reorganization when mitochondrial structures are abundant. Two-fold increases in RNA and in fat body respiration during the first 10 days of adult life suggest that the male fat body increases its general biosynthetic activity after the cellular reorganization (Mannix & Keeley, 1980; Keeley, 1981). We speculate that mitochondriogenesis and CGH-directed cytohaem synthesis enable the exergonic capacity within the adipocytes to meet elevated biosynthetic responsibilities of the fat body in adult male *B. discoidalis*.

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